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Erysipelothrix rhusiopathiae: Association of Spa-type with Serotype and Role in Protective Immunity

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Abstract
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Keywords
Erysipelothrix rhusiopathiae, Surface protective antigen, Erysipelas

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Erysipelothrix rhusiopathiae: Association of Spa-type with serotype and role in protective immunity

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ABSTRACT

A collection of swine, fish, and cetacean *Erysipelothrix rhusiopathiae* strains representing 16 serotypes was analyzed for possession of the three currently recognized surface protective antigen (spa)-types: spaA, spaB, and spaC. Polymerase chain reaction (PCR) assays and Western blotting with a SpaA-specific monoclonal antibody demonstrated that spa-type is not confined to specific serotype groups. In particular, the spa-type of strains of aquatic origin was more variable than those of terrestrial origin, and possessed the distinct ability to express more than one spa. In a cross-protection study, mice immunized with an *E. rhusiopathiae* serotype 2 SpaA-type strain and challenged with various *E. rhusiopathiae* isolates were completely protected against strains exhibiting a single homologous spa, but variably protected against strains possessing a heterologous spa or those harboring more than one spa-type.

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1. Introduction

Erysipelothrix rhusiopathiae is a bacterial organism which causes erysipelas in a variety of mammals and birds, as well as erysipeloid in humans [1]. Erysipelas is commonly associated with swine, and is characterized by urticarial diamond-shaped lesions which can quickly progress to an acute septicemic infection or death. Chronic erysipelas usually follows an acute infection where self-sustaining, destructive pathological changes in the heart valves and joints produce endocarditis and arthritis, respectively [1]. The disease has recently reemerged in the Midwestern United States [2] but also has an economic and epidemiological impact on animal production and handling worldwide. While outbreaks of the disease are reported most often in swine and turkeys, cetaceans are also commonly affected.

The genus *Erysipelothrix* contains two accepted species, *E. rhusiopathiae* which include serotypes 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, 21 and N; *E. tonsillarum* containing serotypes 3, 7, 10, 14, 20, 22 and 23 [3,4]. Two unclassified *Erysipelothrix* groups representing serotypes 13 and 18 are also recognized [4]. *E. rhusiopathiae* is considered to be the pathogenic specie of the genus [5], and killed and attenuated live vaccines are used to prevent disease. Bacterins for the prevention of swine erysipelas are composed of serotype 2 strains [1] which provide effective cross-protection against serotypes 1a, 1b, and 2 [6,7], the most relevant serotypes of swine and turkey erysipelas [1,8]. These bacterin formulations also protect against other *E. rhusiopathiae* serotypes, but often to a lesser degree [6,7,9]. Marine aquaria also rely on swine strain bacterins to protect against cetacean erysipelas [10,11]; however, complete protection is not always attained [11].

The 64–66 kilodalton (kDa) cell surface protein of *E. rhusiopathiae* is responsible for eliciting highly protective antibodies [12–14], and is considered to be the major immunogenic antigen of the specie [15]. The gene encoding the protective 64–66 kDa protein was first sequenced by Makino et al. [16] who named it, surface protective antigen A (*spa*A). Recently, two additional types of *spa*-related genes were detected within *E. rhusiopathiae* and an unclassified serotype 18 of the genus *Erysipelothrix* [17]. Amino acid sequence analysis determined that serotypes 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17 and N possess *spa*A; serotypes 4, 6, 11, 19 and 21 possess *spa*B; and the unclassified serotype 18 possesses *spa*C [17].

The three molecular groups are distinguished by amino acid sequence and are considered antigenically distinct [17]. In active immunization experiments in mice, recombinant Spa antigens were completely protective against virulent *E. rhusiopathiae* strains possessing homologous *spa* [17,18], but variably protective against strains possessing heterologous *spa*-types [17].

In this study, we evaluated a variety of swine, fish, and cetacean *E. rhusiopathiae* strains representing all 16 serotypes, and found that *spa*-type is not confined to specific serotype groups. We then

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analyzed the ability of a bacterin, formulated with an *E. rhusiopathiae* serotype 2 strain expressing *spa*A, to protect mice against a variety of cetacean and fish isolates of known Spa-type.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and challenge preparation

E. rhusiopathiae strains used in this study are listed in Table 1 . *E. rhusiopathiae* strains from R.L. Wood were serotyped at the USDA National Animal Disease Center or National Veterinary Services Laboratories (Ames, IA) and fish and cetacean strains obtained from the John G. Shedd Aquarium (Chicago, IL) were serotyped at the Veterinary Diagnostic Laboratory, Iowa State University College of Veterinary Medicine (Ames, IA).

Cultures were streaked for isolation and grown on 5% bovine blood agar at 37 °C and propagated as previously documented [17]. Strains used for testing in mice were Piquet, Immiayuk, Quitz, Naluark, Kayavak, Large Herring 182, and Large Herring 911. Overnight cultures were adjusted to 74.0%$T \pm 0.2$ in a spectrophotometer set at 600 nm prior to preparing dilutions for injection in mice.

2.2. PCR amplification

Genomic DNA of *E. rhusiopathiae* strains was purified from 1.0 mL of overnight culture using QIAamp® DNA Mini Kit (Qiagen, Valencia, CA). The following primers were designed from the protective domain of *spa*A of *E. rhusiopathiae* strain Fujisawa (GenBank accession AB019124) using Oligo 6 software (Molecular Biology Insights, Inc., Cascade, CO) and custom synthesized (Iowa State University DNA Facility, Iowa State University, Ames, IA): spaA2F, 5′-CCA AAG GGG TAC CAA AGT T-3′, corresponding to position 259-277, and spaA2R, 5′-GAT TCG GGT TTT GAT TGA-3′, corresponding to position 1328-1311. Polymerase chain reaction was performed in a 50 μL reaction mixture that contained final concentrations of one unit Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.2 μM each primer. Samples were subjected to initial denaturation at 94° C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 7 min.

The following primers were synthesized (Iowa State University DNA Facility) and used in PCR assays according to previously described reports: primer 1, 5′-ATG AAA AAG AAA AAA CAC CTA-3′, and primer 2, 5′-CTA TTT TAA ACT TCC ATC GTT-3′, were used to amplify whole *spa*A [17]; primer 3, 5′-ATG AAA AAG AAA AAA CAC CTA TTT CCG AAA GTA-3′, and primer 4, 5′-CTA TTT TAA ACT TCC ATC GTT CTT AAA TGC ATA-3′, were used to amplify whole *spa*B or *spa*C [17]; ERY-1F, 5′-ATC GAT AAA GTG TTA TTG GTG G-3′, and ERY-2R, 5′-CGA GTG TGA ATC CGT CGT CTC-3′, were used to verify the species of *Erysipelothrix* strains [19].

2.3. Sequencing of the spa protective domain

A 1070 base pair (bp) fragment of the protective domain of *spa*A from genomic DNA of E1-6P, Immiayuk, Piquet, Large Herring 182, and Large Herring 911 was amplified by PCR with primer set spaA2F/R. The PCR product from each strain was purified using the MinElute® PCR purification kit (Qiagen) and inserted into the cloning vector pCR®4-TOPO® (Invitrogen) and transfected into OneShot® TOP10 chemically competent *Escherichia coli* (Invitrogen) following the manufacturer's instructions. Plasmids were purified using the PureLink™ Quick Plasmid Miniprep kit (Invitrogen). Both strands of DNA of cloned PCR products were sequenced on a Model 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA) using a primer-walking procedure starting with vector primers T3 and T7. DNA sequences were assembled using the SeqMan program of the DNASTAR software package (DNASTAR Inc., Madison, WI) and translated into amino acid sequence using EMBOSS Transeq [20]. Amino acid sequences were aligned with Spa sequences of *E. rhusiopathiae* strains Fujisawa (GenBank accession AB019124), SE-9 (AB024084), Dolphin E-1 (AB238212), and *Erysipelothrix* strain 715 (AB238210) using the T-Coffee multiple sequence alignment tool [21].

2.4. Antigen and antibody preparation

Surface antigens of *E. rhusiopathiae* were obtained by extraction with Triton X-100 (Sigma–Aldrich, Inc., St. Louis, MO) as previously described [12,14] and stored at −80 °C.

The monoclonal antibody ERHU-B60-91 was produced by vaccinating mice with a bacterin composed of *E. rhusiopathiae* serotype 2 strains SE-9, CN 3461, CN 3342, and AN-4 as previously described [22]. The antibody has been shown to be specific for the protective domain of SpaA (data not shown).

2.5. SDS-PAGE and Western blot

Total protein of each *E. rhusiopathiae* antigen preparation was estimated using the BCA™ Protein Assay (Thermo Scientific, Rockford, IL). Approximately 50 μg of protein per sample per well was separated in NuPAGE® Novex Bis-Tris 4–12% gradient gels (Invitrogen) in MOPS buffer under reducing conditions. Separated proteins were transferred to nitrocellulose membranes and blocked in 20 mM Tris—500 mM NaCl—0.5% Tween 20 (TTBS, pH 7.5). The proteins were probed with the SpaA-specific monoclonal antibody, ERHU-B60-91 [1,22], and visualized with goat-anti-mouse IgG-horseradish peroxidase conjugated antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and 3,3′,5,5′-tetramethylbenzidine (TMB) and TMB Membrane Enhancer (Kirkegaard and Perry Laboratories) substrate solution.

Separated total protein of each sample was visualized by staining duplicate SDS-PAGE gels with SimplyBlue™ SafeStain (Invitrogen) or by staining duplicate nitrocellulose membranes with AuroDye™ Forte (GE Healthcare, Buckinghamshire, UK).

2.6. Animals

CF-1 mice (Charles River Laboratories, Wilmington, MA) 8–10 weeks old were used for each experiment. Mice were provided food and water ad libitum. All management and experimental procedures were performed in accordance with the requirements of the USDA Center for Veterinary Biologics-National Veterinary Services Laboratory Animal Care and Use Committee which conform to provisions of the Animal Welfare Act (Public Laws 85-544 and subsequent amendments).

2.7. Erysipelothrix rhusiopathiae strain lethality testing

The mouse 50% lethal dose (LD_{50}) of each *E. rhusiopathiae* strain was determined by inoculating groups of mice subcutaneously with 0.1 mL of a 10-fold dilution of overnight culture. Mice were housed in isolation and observed for 10 days for clinical signs and mortality. The LD_{50} was calculated for each strain following the method of Reed and Muench [23].

2.8. Cross-protection studies

A working stock of *E. rhusiopathiae* standard reference bacterin IRP 529(05) was prepared in sterile 0.85% saline as recommended

Table 1
Erysipelothrix rhusiopathiae strains and PCR and Western blotting results.

Strain	Serotype	ERY1F/2R	Primer 1/2	spaA2F/R	Primer 3/4	ERHU-B6091
185–204[g]	1a		Y	Y		Y
Fujisawa[f,h]	1a	Y	Y	Y		Y
Marienfelde[g]	1a		Y	Y		Y
Kuniyasu[g]	1a		Y	Y		Y
ME-7[c,g]	1a		Y	Y		Y
E1-6P[f,g]	1a	Y	Y	Y	O	Y
HC-585[g]	1a		Y	Y		Y
Hydrovac[e,g]	1a		Y	Y		Y
DG 1534[g]	1b		Y	Y		Y
EW-2[g]	1b		Y	Y		Y
DG 1966 sp[g]	1b		Y	Y		Y
422-1E1[c,h]	1b		Y	Y		Y
Norden 141[e,g]	2		Y	Y		Y
R32E11[g]	2		Y	Y		Y
FDvac[e,g]	2		Y	Y		Y
J80 smooth[g]	2		Y	Y		Y
10-4[g]	2		Y	Y		Y
RO-12[g]	2		Y	Y		Y
R-2 Franklin[g]	2		Y	Y		Y
T-28[g]	2		Y	Y		Y
CN 3342[e,g]	2		Y	Y		Y
CN 3461[e,g]	2		Y	Y		Y
266[g]	2		Y	Y		Y
Duragen[e,g]	2		Y	Y		Y
Yena-36[g]	2		Y	Y		Y
A1 Japan[g]	2		Y	Y		Y
AN-4[e,g]	2		Y	Y		Y
S-192[g]	2		Y	Y		Y
ER4[g]	2		Y	Y		Y
ER5[g]	2		Y	Y		Y
SE-9[e,g]	2	Y	Y	Y		Y
ATCC 19414[d,h]	2		Y	Y		Y
NF4E1[c,h]	2		Y	Y		Y
Doggerscharbe[i]	4	Y	O	O	O	O
748[g]	4	Y	O	O	O	O
Pécs 67[c,h]	5		Y	Y		Y
P-190[g]	5		Y	Y		Y
Castro S66[g]	5	Y	O	O	O	O
Castro P23[g]	5	Y	O	O	O	O
Castro S52[g]	6	Y	O	O	O	O
P32[g]	6	Y	O	O	O	O
Tuzok[c,j]	6	Y	O	O	O	O
V1227[g]	8		Y	Y		Y
Goda[c,j]	8		Y	Y		Y
P92[g]	8	Y	O	O	O	O
P26[g]	8	Y	O	O	O	O
Kaparek[c,i]	9		Y	Y		Y
IV 12/8[c,h]	11	Y	O	O	O	O
Pécs 52[g]	11	Y	O	O	O	O
Pécs 9[c,h]	12		Y	Y		Y
Castro S61[g]	12	Y	O	O	O	O
Pécs 3597[c,h]	15		Y	Y		Y
Tanzania[c,j]	16		Y	Y		Y
545[c,h]	17		Y	Y		Y
2017[c,h]	19	Y	O	O	O	O
Baño 36[c,k]	21	Y	O	O	O	O
MEW 22[c,g]	N		Y	Y		Y
C2T0 072605[m]	1a	Y	O	O	O	O
Ariel 100101[m]	1b		Y	Y	O	Y
Small Herring 032700[m]	2		Y	Y	O	Y
C1T1 071204[m]	2		Y	Y	O	Y
Capelin 071000[m]	2		Y	Y	O	Y
Large Herring 040700[m]	2		Y	Y	O	Y
Large Herring 030700[m]	4	Y	Y	O	Y	O
Kri 040201[m]	5	Y	O	O	O	O
C2T0 081004[m]	5	Y	O	O	O	O
Capelin 032200[m]	5		Y	Y	O	Y
Large Herring 071000[m]	6	Y	O	O	O	O
Large Herring 073100[m]	6	Y	O	O	Y	O
Large Herring 182[m]	6		Y	Y	Y	Y
A 061101[m]	8	Y	O	O	O	O
Large Herring 072400[m]	8		Y	Y	O	Y
Orange 120301[m]	12	Y	O	O	O	O
Small Herring 061200[m]	15	Y	O	O	O	O
C2T1 083004[m]	21	Y	Y	O	Y	O
Large Herring 911[m]	21		Y	Y	Y	Y

Table 1 (*Continued*)

Strain	Serotype	ERY1F/2R	Primer 1/2	spaA2F/R	Primer 3/4	ERHU-B6091
Orange 021901[m]	21		Y	Y	Y	Y
Piquet 10507[n]	X[a]		Y	Y	O	Y
Quitz 262[n]	[2,15][b]	Y	O	O	O	O
SW50[l]	8	Y	O	O	O	O
Immiayuk 6567[o]	2		Y	Y	O	Y
Kayavak 7122/7155[o]	5	Y	O	O	O	O
Naluark 10797[o]	X[a]	Y	O	O	O	O
ATCC 43339[d,p]	7	O				

Y positive reaction. O negative reaction.

[a] None of the classical serotypes.
[b] Partially identical.
[c] Serotype reference strain.
[d] Type strain.
[e] Vaccine strain.
[f] Challenge strain.
[g] R.L. Wood collection, unknown origin.
[h] R.L. Wood collection, swine origin.
[i] R.L. Wood collection, fish origin.
[j] R.L. Wood collection, bird origin.
[k] R.L. Wood collection, sheep dip origin.
[l] R.L. Wood collection, dolphin origin.
[m] John G. Shedd Aquarium collection, fish origin.
[n] John G. Shedd Aquarium collection, Pacific white-sided dolphin origin.
[o] John G. Shedd Aquarium collection, Beluga whale origin.
[p] R.L. Wood collection, *E. tonsillarum*, swine origin.

by Center for Veterinary Biologics Notice 08-11 [24]. A 1:30 dilution was prepared from the working stock and used to vaccinate a group of 80 mice with a single subcutaneous 0.2 mL dose. Fifteen days after vaccination, a cross-protection study was performed per *E. rhusiopathiae* strain by challenging groups of 10–12 vaccinated mice. Groups of 10 nonvaccinated mice were used as controls and to calculate LD_{50} per strain. Mice were housed in isolation and clinical signs and mortality were observed for 10 days. Challenge doses, given subcutaneously in 0.1 mL, were E1-6P, 1,242 LD_{50}; Piquet, 749 LD_{50}; Immiayuk, 425 LD_{50}; Quitz, 316 LD_{50}; Naluark, 262 LD_{50}; Kayavak, 425 LD_{50}; and Large Herring 182, 368 LD_{50}.

2.9. Statistical methods

Live-versus-dead numbers in the cross-protection studies were compared by the Fisher exact test using a two-tailed *P*-value [25].

3. Results

3.1. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using primers to detect whole *spa*A, whole *spa*B and *spa*C, and the protective domain of *spa*A in 83 *E. rhusiopathiae* strains (Table 1). Using primers 1/2 and spaA2/F, designed to amplify whole *spa*A and the protective domain of *spa*A, respectively, all R.L. Wood strains of serotypes 1a, 1b, 2, 9, 15, 16, 17, and N produced amplification products of appropriate size. Only half of the strains representing serotypes 5, 8, and 12 produced amplicons with both *spa*A-specific primer sets. There was no gene amplification detected in the R. L. Wood strains of serotypes 4, 6, 11, 19, and 21 with either *spa*A primer set.

Production of amplification products with both *spa*A-specific primer sets was variable in fish and cetacean strains of all serotypes. Only strains of serotypes 1b and 2 consistently produced amplification products of expected size with both primers, while only five of 11 fish strains representing the serotypes 5, 6, 8, and 21 produced amplification products. Single strains of serotypes 4 and 21 produced an amplification product with the whole *spa*A primers, but a product was not produced with the *spa*A protective domain primers. Three fish strains representing serotype 1a, 12, and 15, respectively, failed to produce an amplicon with either *spa*A-specific primer set. Only two of six cetacean strains, a serotype 2 Beluga whale strain and a nontypeable dolphin strain, produced amplification products of expected size with both *spa*A-specific primer sets.

Primers 3/4, designed to amplify whole *spa*B and *spa*C, were used to detect those genes from all *E. rhusiopathiae* fish and cetacean strains, as well as R.L. Wood strains which did not react with the *spa*A-specific primers. Six of seven fish strains representing serotypes 4, 6, and 21 produced an amplicon of approximately 1880 bp with the primer set. No amplification product was produced from any cetacean strain or strains from the R.L. Wood collection using the *spa*B/C-specific primer set.

A PCR assay utilizing the *E. rhusiopathiae* species-specific primers, ERY1F/2R, was used to verify the species of strains nonreactive with any of the *spa*-specific primers. An amplicon 2210 bp in length was produced from each strain, confirming that these strains were *E. rhusiopathiae*.

3.2. Protective domain sequence comparison

The protective domain of *spa*A was amplified by PCR from five *E. rhusiopathiae* strains: swine strain E1-6P (serotype 1a); Beluga whale strain Immiayuk (serotype 2); a nontypeable Pacific white-sided dolphin strain Piquet; and two fish strains, Large Herring 182 (serotype 6) and Large Herring 911 (serotype 21). Alignment of the five deduced amino acid sequences found that the 342 amino acids of the protective region ranged from 98.0% to 99.7% identical to the SpaA protective domain sequence of strain Fujisawa (AB019124); 98.5–99.7% identical to the SpaA protective domain sequence of strain SE-9 (AB024084); 51.7–52.8% identical to the SpaB protective domain sequence of strain Dolphin E-1 (AB238212); and 56.7–57.0% identical to the SpaC protective domain sequence of strain 715 (AB238210).

3.3. Expression of spa and reactivity to a SpaA-specific monoclonal antibody

All 83 *E. rhusiopathiae* strains were examined by SDS-PAGE for their expression of a 64–66 kDa protein and its interaction with the SpaA-specific monoclonal antibody, ERHU-B60-91. In SDS-PAGE

Table 2
Mouse LD_{50} of *E. rhusiopathiae* challenge strains.

Strain	CFU per challenge	LD_{50} dose	Strain origin	Serotype
Piquet	2.5×10^2	7.875	Pacific white-sided dolphin	X[a]
Immiayuk	2.7×10^2	7.629	Beluga whale	2
Quitz	4.0×10^2	7.5	Pacific white-sided dolphin	[2,15][b]
Naluark	3.1×10^2	7.419	Beluga whale	X[a]
Kayavak	2.3×10^2	7.629	Beluga whale	5
Large Herring 182	2.1×10^2	7.567	Herring fish	6
Large Herring 911	3.76×10^7	–[c]	Herring fish	21
E1-6P	9.12×10^2	8.094	Swine	1a

[a] None of the classical serotypes.
[b] Partially identical.
[c] No pathogenicity in mice, strain not used in vaccination-challenge study.

analysis, all 83 strains produced a 64–66 kDa protein; however, only those strains which produced an amplicon in PCR with the *spa*A protective domain primers, spaA2F/R, produced a protein recognizable by ERHU-B60-91 (Table 1).

3.4. Strain lethality testing

Pathogenicity data for each strain are listed in Table 2. The swine, fish, and cetacean strains appeared nearly equal in their pathogenicity in mice except for strain Large Herring 911 (serotype 21), which at a dose of 3.76×10^7 colony forming units (CFU), did not kill mice.

3.5. Cross-protection experiment

The experiment was designed to examine whether the standard reference bacterin IRP 529(05), composed of the *E. rhusiopathiae* serotype 2 SpaA-type strain SE-9, could protect mice equally against challenge with cetacean and fish strains of various Spa-types compared to the serotype 1a SpaA-type swine strain, E1-6P (Fig. 1).

Within four days after challenge with E1-6P, all nonvaccinated control mice died while all vaccinated mice survived 10 days when the experiment was terminated. The difference between the vaccinated group and the nonvaccinated group was significant ($P < 0.0001$).

Bacterin IRP 529(05) induced complete protection (100%) against challenge with the SpaA-type dolphin strain Piquet (nontypeable) and the SpaA-type Beluga whale strain Immiayuk (serotype 2). No significant difference was exhibited in the ability of the bacterin to protect mice against Piquet and Immiayuk compared to the E1-6P challenge ($P = 1.0000$). Of 12 vaccinated mice challenged with the SpaA-type Large Herring 182 (serotype 6), only two survived, indicating a significant difference compared to the results of the E1-6P challenge ($P < 0.0001$).

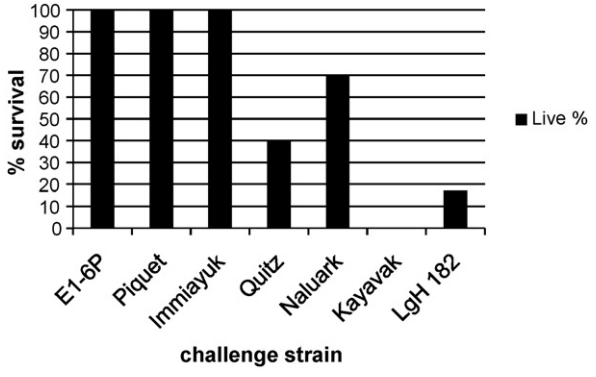

Fig. 1. Percentage of immunized mice that survived challenge with *E. rhusiopathiae* challenge strains E1-6P, Piquet 10507, Immiayuk 6567, Quitz 262, Naluark 10797, Kayavak 7122/7155, and Large Herring 182.

Upon challenge with the non-SpaA Beluga whale strain Naluark (nontypeable), seven of 10 vaccinated mice survived, indicating that partial protection was induced compared to the E1-6P challenge ($P = 0.2105$). The ability of the reference bacterin to protect against challenge with the other non-SpaA-type strains showed significant differences compared to the E1-6P challenge. Four of 10 vaccinated mice challenged with the dolphin strain Quitz (serotype 2, 15) survived ($P = 0.0108$) while none of the 12 vaccinated mice challenged with the Beluga whale strain Kayavak (serotype 5) survived ($P < 0.0001$).

4. Discussion

The Spa proteins of *E. rhusiopathiae* are cell membrane-bound [12–14,16–18] and recognized as the major protective antigen of the species [15]. This present study investigated a wide range of *E. rhusiopathiae* strains and found that (i) *spa*-type is not confined to specific serotype groups, especially in those strains isolated from aquatic animals; (ii) a single *E. rhusiopathiae* strain may possess more than one *spa*-type; (iii) the degree of cross-protection elicited by conventional erysipelas bacterins is dependent on the Spa-status of the *E. rhusiopathiae* challenge strain.

Recently, To and Nagai [17] reported the detection of two additional *Erysipelothrix* Spa-types, SpaB and SpaC. Based on the Spa-type observed in single reference strains, they reported that serotypes 1a, 1b, 2, 5, 8, 9, 12, 15, 16, 17 and N possess *spa*A; serotypes 4, 6, 11, 19 and 21 possess *spa*B; and the unclassified serotype 18 possesses *spa*C. However, earlier reports by both Wood et al. [7] and Takahashi et al. [6] conflict with this organization in that mice vaccinated with an *E. rhusiopathiae* serotype 2 (presumably SpaA) strain were variably protected against certain serotypes currently within the SpaA-group, and completely protected against serotypes placed in a heterologous Spa-group. Although, the role of the 64–66 kDa protein was not realized at the time of those studies, and thus not investigated, it is important to consider the role that Spa-type may have had in those evaluations.

In contrast to previous Spa studies [16,17], this study investigated several strains per serotype. These strains were analyzed by PCR with primers specific for whole *spa*A, the protective domain of *spa*A, and whole *spa*B and *spa*C. Fourteen strains of serotypes described as possessing *spa*A, including, five swine strains representing serotypes 5, 8, and 12; six fish strains representing serotypes 1a, 5, 8, 12, and 15; and three cetacean strains representing serotypes 8, 5, and 2/15, were non-reactive with either set of *spa*A-specific primers. After verifying the species identity of these 14 strains, attempts were made to amplify *spa*B and *spa*C gene products by PCR, but those assays also yielded negative results. Western blot verified that these 14 strains lacked expression of *spa*A when the SpaA protective domain-specific monoclonal antibody, ERHU-

B60-91, failed to detect the protein. The inability to detect *spa*A or its expression product in these particular strains is contrary to the observation reported by To and Nagai [17] which suggests that *spa*-type and serotype are related. In this current study, it appears that these 14 strains have either acquired nucleotide variation of *spa*A or possess a novel *spa*.

The observation that *spa*-type and serotype are not associated was further supported when four fish strains representing serotypes 6 and 21 produced amplicons of correct size in PCR assays using both *spa*A-specific primer sets. Expression of *spa*A was verified by Western blotting when each of these four strains produced an approximately 65 kDa protein that was detected by ERHU-B60-91. In addition, PCR products resulting from amplification with the *spa*A protective domain primers were sequenced, translated into amino acid sequences, and aligned with published sequences representing each Spa-type. These sequences showed high identity to the SpaA of both strains Fujisawa and SE-9, demonstrating that *E. rhusiopathiae* serotypes 6 and 21 (serotypes previously described as possessing *spa*B) can possess and express *spa*A.

In this study, primers designed to amplify *spa*B and *spa*C [17] were used in PCR with all strains of serotypes 4, 6, 11, 19, and 21, all fish and cetacean strains, as well as strains non-reactive with *spa*A-specific primers. Serotype reference strains previously reported as producing a product in PCR with these primers [17] failed to produce an amplification product in this study. Great care was taken to exactly follow the described protocol and several assays were performed to reproduce the published work, but to no avail. However, the *spa*B/C PCR assay did prove successful with six fish strains representing serotypes 4, 6, and 21, which produced amplicons of expected size. These results indicate the possibility that some genetic variation exists between this laboratory's serotype reference strains and those possessed by To and Nagai [17], which prevented the *spa*B/C primers from annealing effectively.

As of yet, *E. rhusiopathiae* strains have only been described as possessing a single *spa*-type. In this study, three fish strains representing serotypes 6 and 21 produced amplicons of expected size in each of the three *spa*-specific PCR assays. Two other fish strains representing serotypes 4 and 21 produced amplicons with the primer sets designed to amplify whole *spa*A and whole *spa*B and *spa*C. Although some cross-reaction is possible due to high sequence similarity of *spa*s in the N-terminal and C-terminal regions, these results imply that a single *E. rhusiopathiae* strain may possess more than one *spa*-type. Many more *E. rhusiopathiae* strains, particularly recent field isolates, must be evaluated to more fully understand the scope and sequence variability of the *spa* family of genes.

Nearly all commercial erysipelas bacterins are formulated with serotype 2 strains which, in swine, offer cross-protection against the most frequently isolated serotypes, 1a, 1b, and 2 [1]. In this study, the only group of *E. rhusiopathiae* strains that consistently possessed and expressed a single *spa*-type were the 33 R.L. Wood strains (mostly clinical swine isolates and vaccine strains) of serotypes 1a, 1b, and 2. These strains all produced a correctly sized amplification product with both *spa*A-specific primer sets, and expressed a protein recognized by ERHU-B60-91; demonstrating that *spa*A is highly conserved in serotypes most often implicated in clinical swine erysipelas. The possession and expression of a homologous *spa*-type likely explain the high rate of cross-protectiveness among these three serotypes.

It has been reported by Lacave et al. [10] that an inactivated *E. rhusiopathiae* swine vaccine strain of serotype 2 can protect mice against challenge with dolphin strains of serotypes 1a, 2, 5, 15, and 21, but only partial protection was exhibited in the long term. Because the Spa-status of the vaccine strain and the challenge strains were not reported in the study, the effect of Spa-elicited cross-protection is unknown. Due to the immunological significance of the Spa family of proteins [15–18,26] and the variable cross-protective nature of the three Spa-types currently recognized [17], it is necessary to evaluate the ability of an erysipelas bacterin to protect against diverse *E. rhusiopathiae* challenge strains. In this present study, the Spa-type of the *E. rhusiopathiae* bacterin strain and challenge strains were investigated prior to testing in mice.

Considering To and Nagai's prior cross-protection study [17], it was not surprising that vaccinated mice were completely protected against challenge strains possessing a single homologous SpaA and variably protected against those strains with a heterologous Spa-type. However, it was interesting that protection against Large Herring 182, a serotype 21 fish strain possessing and expressing *spa*A and possessing at least one other *spa*-type, was incomplete; only 17% of the vaccinated mice survived challenge against Large Herring 182. This result suggests that antibody to SpaA is not sufficient to protect against an *E. rhusiopathiae* strain that co-expresses another *spa*-type. In this study, the *spa*-type observed in marine animal *E. rhusiopathiae* isolates was more variable than expected, and may be contributing to the incomplete protection seen in cetaceans vaccinated with commercial swine erysipelas bacterins.

Outbreaks of erysipelas have recently been reported in the Midwestern United States among vaccinated and nonvaccinated swine [2]. Although the outbreaks in these cases were ultimately attributed to inappropriate vaccine management, the variable cross-protective nature of the Spa proteins may reveal the cause of some vaccines' failure to protect. In future erysipelas cases, a comparison of the *spa*-type(s) of the vaccine and challenge strains should be conducted.

The serotype classification of *Erysipelothrix* is based on soluble peptidoglycan antigens of the cell surface [1], and without international guidelines, serotyping results are often inconsistent among laboratories [14]. Molecular-based systems are proving to be a more efficient and reliable method of organizing *E. rhusiopathiae* strains [5] and assimilation to a new system is anticipated. Because of their significance, a new classification scheme based on *spa* may be a worthwhile approach.

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References

[1] Wood RL, Henderson LM. Erysipelas. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ, editors. Diseases of swine. ninth ed. Ames, IA: Blackwell Publishing; 2006. p. 629–38.
[2] Opriessnig T, Hoffman LJ, Harris DL, Gaul SB, Halbur PG. *Erysipelothrix rhusiopathiae*: genetic characterization of midwest US isolates and live commercial vaccines using pulsed-field gel electrophoresis. J Vet Diagn Invest 2004;16:101–7.
[3] Takahashi T, Fujisawa T, Benno Y, Tamura Y, Sawada T, Suzuki S, et al. *Erysipelothrix tonsillarum* sp. nov. isolated from tonsils of apparently healthy pigs. Int J Syst Bacteriol 1987;37(2):166–8.
[4] Takahashi T, Fujisawa T, Tamura Y, Suzuki S, Muramatsu M, Sawada T, et al. DNA relatedness among *Erysipelothrix rhusiopathiae* strains representing all twenty-three serovars and *Erysipelothrix tonsillarum*. Int J Syst Bacteriol 1992;42(3):469–73.
[5] Takahashi T, Fujisawa T, Umeno A, Kozasa T, Yamamoto K, Sawada T. A taxonomic study on *Erysipelothrix* by DNA-DNA hybridization experiments with numerous strains isolated from extensive origins. Microbiol Immunol 2008;52:469–78.

[6] Takahashi T, Takagi M, Sawada T, Seto K. Cross protection in mice and swine immunized with live erysipelas vaccine to challenge exposure with strains of *Erysipelothrix rhusiopathiae* of various serotypes. Am J Vet Res 1984;45(10):2115–8.
[7] Wood RL, Booth GD, Cutlip RC. Susceptibility of vaccinated swine and mice to generalized infection with specific serotypes of *Erysipelothrix rhusiopathiae*. Am J Vet Res 1981;42(4):608–14.
[8] Bricker JM, Saif YM. Erysipelas. In: Calnek BW, editor. Diseases of poultry. tenth ed. Ames, IA: Iowa State University Press; 1997. p. 302–13.
[9] Sawada T, Takahashi T. Cross protection of mice and swine inoculated with culture filtrate of attenuated *Erysipelothrix rhusiopathiae* and challenge exposed to strains of various serovars. Am J Res 1987;48(2):239–42.
[10] Lacave G, Cox E, Hermans J, Devriese L, Goddeeris BM. Induction of cross-protection in mice against dolphin *Erysipelothrix rhusiopathiae* isolates with a swine commercial vaccine. Vet Microbiol 2001;80:247–53.
[11] Middlebrooks BL, Jones JC, Patterson RA. Application of ELISA methodology for detection of *Erysipelothrix rhusiopathiae* antibody titers in cetaceans. In: Pfeiffer C, editor. Molecular and cell biology of marine mammals. Melbourne, FL: Krieger Publishing Co; 2002. p. 193–204.
[12] Lachmann PG, Deicher H. Solubilization and characterization of surface antigenic components of *Erysipelothrix rhusiopathiae* T28. Infect Immun 1986;52(3):818–22.
[13] Groschup MH, Cussler K, Weiss R, Timoney JF. Characterization of a protective protein of *Erysipelothrix rhusiopathiae*. Epidemiol Infect 1991;107:637–49.
[14] Galán JE, Timoney JF. Cloning and expression in *Escherichia coli* of a protective antigen of *Erysipelothrix rhusiopathiae*. Infect Immun 1990;58(9):3116–21.
[15] Imada Y, Mori Y, Daizoh M, Kudoh K, Sakano T. Enzyme-linked immunosorbent assay employing a recombinant antigen for detection of protective antibody against swine erysipelas. J Clin Microbiol 2003;41(11):5015–21.
[16] Makino S-I, Yamamoto K, Murakami S, Shirahata T, Uemura K, Sawada T, et al. Properties of repeat domain found in a novel protective antigen, SpaA, of *Erysipelothrix rhusiopathiae*. Microb Pathog 1998;25:101–9.
[17] To H, Nagai S. Genetic and antigenic diversity of the surface protective antigen proteins of *Erysipelothrix rhusiopathiae*. Clin Vacc Immunol 2007;14(7):813–20.
[18] Imada Y, Goji N, Ishikawa H, Kishima M, Sekizaki T. Truncated surface protective antigen (SpaA) of *Erysipelothrix rhusiopathiae* serotype 1a elicits protection against challenge with serotypes 1a and 2b in pigs. Infect Immun 1999;67(9):4376–82.
[19] Yamazaki Y. A multiplex polymerase chain reaction for discriminating *Erysipelothrix rhusiopathiae* from *Erysipelothrix tonsillarum*. J Vet Diagn Invest 2006;18:384–7.
[20] EMBOSS Transeq Tool. http://www.ebi.ac.uk/Tools/emboss/transeq/ Accessed September 9–11, 2008 and December 18–19, 2008.
[21] T-Coffee Multiple Sequence Alignment Tool. http://www.ebi.ac.uk/Tools/t-coffee/ Accessed September 9–11, 2008 and December 18–19, 2008.
[22] Henderson LM. A monoclonal antibody specific for a putative protective immunogen of *Erysipelothrix rhusiopathiae*. Thesis Paper 4; 1996.
[23] Reed LJ, Muench H. A simple method for estimating fifty percent endpoints. Am J Hyg 1938;27:493–7.
[24] Center for Veterinary Biologics Notice 08-11. Reinstatement of *Erysipelothrix rhusiopathiae* Standard Reference Bacterin, IRP 529(05). http://www.aphis.usda.gov/animal_health/vet_biologics/vb_notices_2008.shtml Accessed September 23; 2008.
[25] Fishers exact test. http://www.langsrud.com/fisher.htm. Accessed December 22; 2008.
[26] Shimoji Y, Mori Y, Fischetti VA. Immunlogical characterization of a protective antigen of *Erysipelothrix rhusiopathiae*: identification of the region responsible for protective immunity. Infect Immun 1999;67(4):1646–51.